REMARKS

Applicants acknowledge Examiner's amendments made as indicated on page 2 of the January 28, 2003 Office Action.

Claim Objections

Disclosure was objected to because page following page 12 lacked page number, the words were partially cut off and the bottom margin was less that ¾ of an inch. In response to these objections, applicants submit a replacement page of page 13, wherein the page number and the margins have been amended to comply with 37 CFR 1.52(b)(i). No new matter is introduced by these clerical amendments and, therefore, entry of the replacement sheet for page 13 is respectfully requested.

Claim 21 was objected because the word "nontoxic" was misspelled as "no-toxic." Applicants have amended claim 21 to correct the spelling mistake. Accordingly, no new matter is introduced by this clerical amendment and its entry is respectfully requested.

Claim Rejections

Claims 16-22 were rejected under 35 U.S.C. § 112, 1st paragraph. Examiner contends that the claims as they stand read on treating any tumor in any living host with any exogenous protein.

Applicants respectfully disagree.

However, to expedite prosecution, applicants have amended the claims to claim a "mammal", instead of a "living host." The amendment is supported by the specification as a whole and particularly, for example, the first paragraph of the Background and on page 2, line 15. No new matter has been added by this amendment and its entry is respectfully requested.

In addition, Applicants respectfully submit that the claim language must not be analyzed in vacuum but in light of the content of the specification and teachings of the prior art to one skilled in the art. See In re Moore, 58 CCPA 1042, 439 F.2d 1232, 169 USPQ 236 (1971). Applicants submit that the claim language clearly defines that the exogenous protein is associated with treatment of tumor in stating "specific protein product which is expressed by said modified neural stem cells to treat the tumor cells." A person skilled in the art at the time of filing the application would have been well aware of the proteins which are useful in treating tumors. Such proteins are further exemplified on page 7, lines 19-22, and page 8, lines 1-2.

Examiner further argues that in the field of gene therapy, "no successful outcome has been achieved" and that therefore, the present invention is not enabled in light of the specification.

Applicants respectfully disagree.

It appears that the examiner has chosen to emphasize what the press is most interested in reporting, and that is the disasters and problems in a few well publicized cases. Applicants respectfully submit that of the currently ongoing about 23 gene therapy clinical trials in the United States only, at least 3 are reportedly already on Phase III stage, which clearly indicates

success of such programs (http://www.clinicaltrials.gov). In Phase III studies in general, the study drug or treatment is given to large groups of people (1,000-3,000) to confirm its effectiveness, monitor side effects, compare it to commonly used treatments, and collect information that will allow the drug or treatment to be used safely. Of these clinical trials, for example, Ad5CMV-p53 gene therapy was already on Phase I on March 2, 2001 (Introgen Therapeutics, Inc. (ticker: ingn, exchange: NASDAQ) News Release - 3/2/01, www.introgen .com). Moreover, although FDA imposed a temporary restriction on gene therapy trials using retroviral vectors in January 2003, already in March 5, 2003 FDA's Biological Response Modifiers Advisory Committee recommended that the agency allow gene therapy clinical trials involving children with X-linked severe combined immunodeficiency (X-SCID) syndrome to proceed if no other treatment is available (*FDA Daily Bulletin,* March 5, 2003 and *Washington Drug Letter,* March 10, 2003). Further, in June 16, 2003, FDA lifted its hold on the Enzo Biochem Inc. clinical trial relating to HIV gene therapy (http://healthcenter.bna.com). Therefore, Applicants submit that all these developments clearly argue against a generalized contention of "complete lack of success" of gene therapy trials. Applicants respectfully submit that the Examiner's general contention that "the prior art as of the effective date of the present application shows complete lack of success based on gene therapy" appears to be based on incomplete set of facts. Specifically, when a disease, such as many tumors, does not have any effective cure, any methods or improvements for the treatment are welcomed by the patients, doctors as well as the regulatory bodies evaluating the treatments. For the US PTO to deny the

possibility of commercial development of such treatments by denying their patentability would be unfair, if not unethical, considering the hundreds of thousands of patients desperate for improvement of their chance for cure or even just a slight improvement in the quality of their remaining life.

Furthermore, on page 5, the Examiner listed alleged obstacles that arguably had not been addressed in the present specification. These obstacles included: sustained gene expression, clinical efficiency of gene therapy, and unpredictive animal models.

First, Applicants respectfully submit that the Examiner's comments concerning sustained gene expression are not applicable to the current claims, because treatment of tumors is not intended to last a long time unlike in the treatment of inherited diseases such as cystic fibrosis, haemophilias and X-SCID.

Second, the Examiner cited published references such as Palu et al. (1999) *J. Biotechnology* 68: 1-13; Verma et al. (1997) *Nature* 389:239-242; and Park et al. (2002) *Gene Therapy* 9:613-624. All these references relate to **clinical and therapeutic efficacy**. That is **not the standard** that is to be used in the PTO. *In re Brana*, 51 F.3d 1560, 1568, 34 USPQ 2d 1437 (Fed. Cir. 1995). Further, reduction to practice of a patentable invention does not require that the invention be in a commercially satisfactory stage of development. *Scott v. Finney*, 34 F.3d 1058, 32 USPQ 2d 1115 (Fed. Cir. 1994). Applicants further emphasize that in the case of several cancers, efficacy in treatment is not necessarily even determinative of a successful treatment - a

success in these treatments may be measured by a slight improvement of the quality of a patient's life.

Third, it is well known that a rat glioma model (D74), which applicants describe, e.g., in the Example 2 of the present application, is one of the best glioma animal models, wherein the problem raised by Noble, i.e. the invasiveness of the cells, has been resolved. It is well known to a skilled artisan that the D74 rat glioma has been refractory to a variety of therapeutic modalities and its invasive pattern of growth and uniform lethality makes it make a particularly attractive model to test new therapeutic modalities (see, e.g. Barth, (1998) *J Neurooncol.* Jan;36(1):91-102).

The Examiner contends that the specification provides "little or no guidance to support the claimed invention."

Applicants respectfully disagree and refer to the discussion provided above wherein Applicants submit that clinical and therapeutic efficacy are not the standard that is to be used in the PTO. *In re Brana*, 51 F.3d 1560, 1568, 34 USPQ 2d 1437 (Fed. Cir. 1995). Further, reduction to practice of a patentable invention does not require that the invention be in a commercially satisfactory stage of development. *Scott v. Finney*, 34 F.3d 1058, 32 USPQ 2d 1115 (Fed. Cir. 1994).

Applicants respectfully submit that, in addition to the specification as a whole, the specific examples, wherein applicants demonstrate the working principles of the present invention by using well established animal models give ample support for the method of the

invention to treat tumors using the genetically modified mammalian neural stem cells (for rat model, see, Example 2, 1st par., and mouse model, see e.g. Fig. 2D).

As further evidence that the tumor treatment of the present invention is, indeed, successful, Applicants submit a manuscript authored by the inventors (Appendix I). The findings set forth in the manuscript support the use of neural stem cells as an effective delivery vehicle to target and disseminate therapeutic agents to invasive tumors of neural and non-neural origin, both within and outside the brain.

In light of the above, applicants respectfully submit that the rejection under 35 U.S.C. § 112, 1st paragraph be withdrawn.

In view of the foregoing amendment, Applicants respectfully submit that all claims are in condition for allowance. Early and favorable action is requested.

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Respectfully submitted,

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Intravascular Delivery of Neural Stem Cells to Target Intracranial and Extracranial Tumors of Neural and Non-Neural Origin

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Running headline: Brown and Yang *et al.*; NSCs target tumors

Key words: neural stem cells, vascular delivery, tumors, gene therapy

ABSTRACT

The remarkable migratory and tumor-tropic capacities of neural stem cells (NSCs, and/or neuroprogenitor cells) represent a potentially powerful approach to the treatment of invasive brain tumors, such as malignant gliomas. We have previously shown that whether implanted directly into or at distant sites from an experimental intracranial glioma, NSCs distributed efficiently throughout the main tumor mass and also tracked advancing tumor cells, while stably expressing a reporter transgene. As therapeutic proof-of-concept, NSCs genetically modified to produce the pro-drug activating enzyme cytosine deaminase (CD), effected an 80% reduction in the resultant tumor mass, when tumor animals were treated with the systemic pro-drug, 5-Fluorocytosine (Aboody et al, 2000). We now extend our findings of the tumor-tropic properties of NSCs, by investigating their capacity to target both intracranial and extracranial tumors, when administered into the peripheral vasculature. We furthermore demonstrate their capacity to target extracranial non-neural tumors such as prostate cancer and malignant melanoma. Well-characterized NSCs (lacZ and/or CD positive) were injected into the tail vein of adult nude mice with established experimental intracranial and/or subcutaneous flank tumors of neural and non-neural rigin. The time course and distribution of NSCs within the tumor, and internal organs, was assessed in various models. Resulting data suggest that NSCs can localize to various tumor sites when injected via the peripheral vasculature, with little accumulation in normal tissues. Our findings suggest the novel use of intravascularly administered NSCs as an effective delivery vehicle to target and disseminate therapeutic agents to invasive tumors of neural and non-neural origin, both within and outside of the brain.

OVERVIEW SUMMARY

We have previously demonstrated the inherent migratory and tumor-tropic properties of neural stem cells (NSCs), and their potential utility as delivery vehicles for targeting therapeutic genes/bioactive agents to intracranial glioblastomas. Studies have shown that NSCs are able to target brain tumor cells, irrespective of tumor size or location, and stably express a therapeutic transgene to effect an anti-tumor response. We now present studies further extending these migratory, tumor-tropic properties of NSCs to target both brain and systemic tumors, of neural and non-neural origin, when injected via the peripheral vasculature. NSCs stably transduced to express a reporter or therapeutic transgene localize to experimental intracranial and extracranial subcutaneous flank tumors, when injected via the tail vein, with little accumulation in normal tissues. Our findings suggest the novel, clinically relevant use of intravascularly administered NSCs as an effective delivery vehicle to target and disseminate therapeutic agents, to invasive tumors within and outside the brain — both of neural and non-neural origin.

INTRODUCTION

The inherent migratory and tumor-tropic characteristics of neural stem cells (NSCs, also termed neural progentior cells) represent a potentially powerful approach to the treatment of cancer. NSCs are primordial, uncommitted cells postulated to differentiate into diverse neuronal and glial cell types (Flax *et al.*, 1998; Gage, 2000; Watt and Hogan, 2000). We previously demonstrated the ability of NSCs (C17.2 murine line) to migrate toward and distribute throughout experimental intracranial gliomas (a prototype for brain tumors in general), when injected into adult rodent brain (Aboody et al., 2000). When implanted directly into an established intracranial glioma, NSCs distributed efficiently throughout the main tumor mass, and tracked tumor cells advancing into the surrounding tissue, while stably expressing a reporter transgene. Furthermore, when injected into brain sites distant from the main tumor bed -- including the contra-lateral hemisphere or lateral ventricle -- NSCs migrated through normal appearing brain tissue to specifically target the tumor cells (Aboody et al., 2000). We also demonstrated that NSCs, genetically modified *ex vivo* to express the pro-drug activating enzyme, cytosine deaminase (CD) (Huber et al., 1994), displayed similar migratory properties in relation to rodent gliomas and following treatment with the pro-drug 5-fluorocytosine (5-FC), resulted in a significant reduction in tumor mass in rodent brain (Aboody et al., 2000). These encouraging results suggest the potential use of inherently migratory NSCs as a delivery vehicle for more effectively targeting therapeutic genes and vectors to infiltrating intracranial brain tumors.

We now present evidence that NSCs injected via the peripheral vasculature can target transgenes to a range of tumor types in various locations of the body. Specifically, these studies evaluate the migratory and tumor-targeting abilities of intravascularly delivered NSCs to

different tumor types, including those of neuroepithelial (glioma, neuroblastoma) and non-neuroepithelial (prostate cancer, melanoma) tumors, established in either the brain or subcutaneous flank of adult nude mice. The distribution of NSCs in both tumors and normal tissues was determined at various timepoints after injection using histochemical and immunocytochemical staining. Results suggest that NSCs provide an innovative targeting and delivery system, whereby systemically administered, genetically modified cells can direct therapeutic agents to both intracranial and extracranial tumors — including tumors of neural and non-neural origin.

Cell culture

Murine cell lines used were established by retroviral transduction as previously described (Pear et al., 1993), neomycin or puromycin selection, and sub-cloning by limiting dilution (Aboody et al., 2000). The C17.2 clonal murine NSC line constitutively expressing lacZ (Martinez-Serrano, 1998) (Snyder et al., 1997) was kindly provided by Dr. Evan Snyder (Department of Neurology, Harvard Medical School, Boston, MA). The clonal C17.CD2 line was derived from C17.2 cells by retroviral transduction with an expression cassette for cytosine deaminase and puromycin resistance genes, and stably expresses both lacZ and cytosine deaminase (Aboody et al., 2000). Cells were maintained in DMEM supplemented with 10% fetal calf serum (FCS), 5% horse serum (HS), 1% glutamine, and 50 units per ml penicillin/50 μg per ml streptomycin at 37¡C in 5% CO_2/95% air at 100% humidity. SMHM3 human melanoma cells, PC3 human prostate tumor cells (ATCC), SH-SY5Y human neuroblastoma cells (ATCC), U-251 human glioma cells (kindly provided by Dr. Daryl Bigner, Duke Medical Center), PAE/KDR porcine aortic endothelial cells stably transfected with KDR (a gift from Dr. Lena Claesson-Welsh, Ludwig Institute, Uppsala Sweden), and CNS-1.GFP rat glioma cells (Kruse et al., 1994; Aboody-Guterman et al., 1997) were maintained in DMEM supplemented with 10% FCS, 1% glutamine, and 50 units per ml penicillin/50 μg per ml streptomycin under routine culture conditions. All cell lines were harvested by gentle resuspension in a trypsin-EDTA solution (0.05% trypsin, 0.5 mM EDTA, GIBCO) prior to either implantation or injection. To obtain conditioned media, cell lines were grown on 35 mm plates to 80% confluency. Cultures were rinsed three times with serum-free medium and incubated for another 48 hours in serum-

free DMEM supplemented with 1% glutamine, 1% non-essential amino acids, and 50 units per ml penicillin/50 µg per ml streptomycin under routine culture conditions. Media were collected, cleared by centrifugation for 10 minutes at 600g and stored at -80¡C.

Migration assay

Neural stem cell migration in response to conditioned media was assessed using a modified Boyden chamber assay as previously described (Schmidt et al., 1999). Briefly, quadruplicates of the chemoattractants were added to the lower wells of a 96-well modified Boyden chamber (Neuroprobe, Cabin John, MD), and wells were covered with an 8- m pore size Nucleopore filter coated with Fibronectin (Sigma). NSCs were then suspended at 2.5 X 10^4 cells in 50 l of serum free DMEM medium containing 0.1% bovine serum albumin and seeded into the upper wells. After incubation for 6 hr at 37¡C, non-migrated cells were scraped off the upper side of the filter and filters were stained with Diff Quick (Dade, Switzerland). Nuclei of migrated cells were counted in five high power fields using a 20X objective with a calibrated ocular grid. Values from two independent experiments were expressed as the mean +/- standard error in percentage of the control migration (=100%). The basal migration rate was assessed in response to serum free DMEM containing 0.1% bovine serum albumin only.

Tumor formation

To establish experimental intracranial tumors, tumor cells were implanted into the brains of immuno-deficient adult female nude mice using standard procedures as previously described (Rainov et al., 1999; Aboody et al., 2000). Briefly, animals were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg Ketalar) and xylazine (5 mg/kg Atravet) and

received stereotactically-guided injections of 5 X 10^4 tumor cells in 1 µl of PBS (via a 30 gauge Hamilton syringe over 3-5 min) into the forebrain (~2 mm lateral and 1 mm anterior to bregma, at a 2 mm depth from the dural surface). After retracting the needle over a 3-5 min period, the burr hole was occluded with bone wax, and the scalp incision sutured closed. To establish experimental extracranial tumors, 5-10 X 10^6 tumor cells in 300 µl of PBS were injected subcutaneously into the flanks of adult female nude mice.

NSC peripheral vascular injection

Cultured NSCs were resuspended at a final concentration of 2-3 X 10^6 cells in 200 µl PBS. Animals, bearing previously implanted established tumors, were anesthetized (ketamine/xylazine as above), and 200 µl NSC suspension was injected into the tail vein using a 30 gauge syringe. Animals were sacrificed at various time points (0-7 days later) by an overdose of anesthetic, and tumors and organs (including brain, liver, spleen, kidneys, lungs) were harvested for histology. The tissues were post-fixed overnight in 4% paraformaldehyde in PBS and then cryoprotected in a 30% sucrose/PBS solution. Cryostat sections were prepared as 10 m serial sections placed onto glass slides for subsequent histological and/or immunocytochemical analysis.

Histochemical and immunocytochemical analysis

Cryostat sections were processed for 5-bromo-4-chloro-3-indolyl §-D-galactoside (X-gal) histochemistry, as previously described (Turner and Cepko, 1987). Briefly, sections were stained for *Escherichia coli* §-galactosidase (§ gal) activity to identify foreign transgene (lacZ)-expressing, blue-staining donor-derived cells, and the sections were then counterstained with

neutral red to detect distinctively dark red, elongated tumor cells. Adjacent sections were prepared for dual-filter immunofluorescence using a polyclonal anti-§ gal antibody (Chemicon) detected with a Texas Red-conjugated secondary antibody to mouse IgG (1:1000, Vectastain) to identify lacZ-expressing cells as red, and a monoclonal anti-GFP antibody (1:500, Clontech) detected with a FITC-conjugated secondary antibody to mouse IgG (1:1000, Vectastain) to recognize CNS-1.GFP tumor cells as green. To delineate blood vessels, antibodies to the endothelial cell marker proteins, polyclonal von Willebrand factor (anti-vWF, Dako) and monoclonal CD31 (anti-CD31, Sigma) were used to establish a dual labeling system whereby FITC-labeled cells could be easily identified in relationship to the blood vessel walls within each specimen. To diminish auto-fluorescence, some sections were pre-incubated in a 0.1% sodium borohydride/PBS solution prior to incubation in blocking solution (5% BSA + 3% Normal Donkey Serum + 0.1% Triton - X100 in 1 X PBS). Incubations with primary antibody (anti-vWF at 1:600 or CD31 at 1:100) were performed overnight and detection was done using biotinylated secondary antibody-Texas Red streptavidin conjugates. For immunoperoxidase staining, a biotinylated anti-fluorescein antibody was used, and incubation with secondary antibody was followed by detection of horseradish peroxidase activity, according to standard Vectastain Elite kit protocols. Hematoxylin or DAPI counter-staining was done after the final PBS rinse. After the final steps in either protocol, the slides were mounted for peroxidase-based detection with Crystal Mount (Electron Microscopy Sciences) and for fluorescence with Fluorescent Mounting Medium, Dako), and examined by either light or fluorescence microscopy. NSCs were quantitated by computer image analysis, counting cells in 10 representative sections from each tissue type collected (NSCs were counted in at least 6 random high power fields per section).

RESULTS

Neural stem cell migration in response to conditioned media from glioma and non-neural tumor cell lines

Migratory capabilities of the neural stem cells were examined in an *in vitro* migration assay to determine if NSCs display an affinity for non-neural, as well as neural tumor cells. We utilized the modified Boyden chamber assay to evaluate the effects of conditioned media from glioma cell lines (human U251 and rat CNS-1.GFP) and non-neural tumor cell lines (mouse melanoma B16-F10 and human prostate cancer PC3) (Figure 1). All tumor cell line conditioned media significantly stimulated the directional migration of the NSCs compared to the control. As expected, NSCs showed a significant affinity for the glioma cell lines U251 and CNS-1.GFP with 262.23 – 9% ($p< 0.05$, t-test) and 172.09 – 6.7% ($p< 0.05$, t-test) of the control migration, respectively. However, maximal stimulations were found in response to the human prostate cancer cell line (PC3) with 312.6 – 23.75% ($p< 0.05$, t-test) and the murine melanoma cell line (B16/F10) with 305.9 – 25.96% ($p< 0.05$, t-test). To evaluate if NSCs display an affinity to endothelial cells we used PAE/KDR cells which is a known cell line used for angiogenic assays (Joki *et al.*, 2001) and displays biological characteristics similar to those of human umbilical vein endothelial cells (Waltenberger et al., 1994). Conditioned medium from PAE/KDR did not induce a significant NSC migration (109.79 – 11.6%).

Intravascularly delivered transgene-expressing NSCs display tropism for extracerebral neural tumors

To determine if intravenous injection of NSCs would result in targeting of experimental neural tumors outside the brain, 5-10 X 10^6 CNS-1.GFP rodent glioma [Fig. 2A-B], human U-251

glioma [Fig. 2C], or SH-[redacted]5Y human neuroblastoma [Fig. 2E-F] cells were implanted subcutaneously in the flanks of adult nude mice. Two to four weeks later, when flank tumors were well established ($\geq$ 2mm), 2-3 X 10^6 NSCs were injected via the tail vein. To track NSC cells *in vivo*, clonal lines were engineered to stably express the lacZ reporter gene (C17.2), or both the lacZ reporter gene and the pro-drug-activating enzyme, cytosine deaminase (C17.CD2). On Day 4 following NSC administration, the animals were sacrificed and the tumor mass, liver, kidneys, spleen, heart and brain were harvested, post-fixed in 4% paraformaldehyde, and processed histologically, as previously described. Sections of CNS-1 tumor (10-12 m), stained with X-gal and neutral red, revealed donor C17.2 NSCs distributed within the tumor mass, but not in surrounding normal-appearing tissue (Fig. 2A). Immunofluorescent staining of adjacemt sections with Texas red conjugated β-gal antibody confirmed the X-gal results (Fig. 2B). Similar findings were observed with C17.2 NSCs in the U-251 subcutaneous tumor model (Fig. 2C) and C17.CD2 NSCs in the SH-SY5Y subcutaneous tumor model (Fig. 2D-F). Distribution of NSCs within tumors ranged from patchy to widespread. Such staining was not found in tumors in the absence of NSC injection. When vehicle alone (PBS with no tumor cells) was injected subcutaneously, NSCs did not migrate to the site of injection. From these findings we conclude that following peripheral vein injection, donor NSCs were able to localize to and infiltrate neural tumors outside the brain, in a subcutaneous tumor model.

Intravascularly delivered NSCs localize to tumor vasculature

To further investigate the specific location of the NSCs in relation to tumor vasculature, 10-12 m subcutaneous tumor sections were processed for double immunofluoresence using a Texas red conjugated anti-CD31 antibody to identify the blood vessels, and FITC-conjugated anti-§gal

antibody to identify the donor NSCs. Figure 3 shows a representative tumor section in an animal with a subcutaneous U-251 tumor mass, 4 days following NSC tail vein injection. The tumor is highly vascular showing an extensive network of microvessels (red). NSCs (green) are distributed not only inside or in the near vicinity of the vascular space but also throughout the tumor parenchyma indicating an active transendothelial migration towards the tumor cells.

Intravascularly delivered cytosine deaminase-expressing NSCs (C17.CD2) display tropism for extracerebral tumors

To show that NSCs containing a therapeutic gene retain their ability to target subcutaneous tumors via intravascular delivery, and to determine how soon after intravascular injection NSCs could be found in the tumors, 4-5 X 10^6 SH-SY5Y human neuroblastoma cells were implanted subcutaneously into the left and right flanks of adult nude mice. Three weeks later, when the bilateral tumors were well established, the animals were anesthetized and the left tumor mass surgically removed (as a control) and the skin incision closed. Immediately after, 2-3 X 10^6 C17.CD2 NSCs were injected via the tail vein. The animals were sacrificed by anesthetic overdose approximately 30 min after injection, and the right flank tumor mass and organs harvested, post-fixed and processed histologically as above. Tissue sections were stained with X-gal and neutral red to examine donor NSC distribution within the experimental subcutaneous tumor mass. X-gal staining revealed donor NSCs all along the outer border of the tumor mass, typically in areas of neovasculature, and not in surrounding normal-appearing tissue (Fig 4B, D-G, black arrowheads and arrows). No such staining was observed in the left tumor mass that had been removed immediately prior to NSC injection (Fig 4A,C). Immunofluorescent staining with antibodies to β-gal (to identify NSCs) was also performed to independently

confirm the presence of d -derived cells (data not shown). Thus, it appears that NSCs are strongly enriched in areas of tumor neovasculature.

Intravascularly delivered NSCs distribute to both intracranial and subcutaneous experimental tumors within the same animal

We next wanted to determine whether one injection of intravascularly delivered NSCs could target both intracranial and extracranial tumors within the same animal. Eight X 10^4 human U-251 glioma cells were implanted into right frontal lobe and 5-10 X 10^6 human U-251 glioma cells were implanted into the right subcutaneous flank of adult nude mouse. After tumors were established (8 days after frontal lobe implantation), 2-3 X 10^6 NSCs were injected into the tail vein. Four days following NSC tail vein administration, the animals were sacrificed, and tumor and organs harvested, post-fixed in 4% PFA, cryosectioned, and stained. Low (Fig. 5A) and high power (Fig. 5B) intracranial tumor sections processed for immunofluorescence using Texas red-conjugated anti-§-gal antibody (donor NSCs) and DAPI counterstain to delineate cell nuclei (white arrowheads delineate 2 frontal lobe tumor sites). Low (Fig. 5C) and high power (Fig. 5D) subcutaneous flank tumor sections processed for immunofluorescence using Texas red-conjugated anti-§-gal antibody (donor NSCs) and DAPI counterstain. We note NSCs (red) distributed within both the brain and subcutaneous tumor masses, indicating that intravascularly delivered NSCs have the potential to simultaneously target multiple tumor sites within the same animal.

Intravascularly delivered CD expressing NSCs localize to non-neural subcutaneous tumors. We further investigated the tumor-tropic capacities and rapidity of co-localization of NSCs to tumor in a non-neural tumor model. To determine if intravascularly delivered NSCs

would display the same t[redacted]r-tropic capabilities towards non-neural tumors located in the periphery, we established non-neural subcutaneous flank tumors. 5-10 X 10^6 human melanoma cells were implanted subcutaneously into the left flank of adult nude mice. Three weeks later, when the tumors were well established, 2-3 X 10^6 NSCs (C17.CD2) were injected into the tail vein. The animals were sacrificed 30 min later and subcutaneous tumor mass and organs harvested. Cryosections (10-12 micron) were processed for immunofluoresence using Texas red conjugated anti-§gal antibody to identify donor NSCs. In a representative animal, NSCs were found distributed throughout the subcutanenous melanoma mass (Fig. 6A-B). These results suggest that intravascularly delivered NSCs display similar tropism for non-neural subcutaneous experimental tumors even within 30 minutes of administration.

Distribution of NSCs through internal organs 4 days after NSC tail vein injection

On Day 4 after intravascular injection of NSCs into tumor-bearing and control mice, the distribution of these cells was assessed in various internal organs. The animals were sacrificed and organs harvested and processed as described above. Representative tissue sections of spleen stained with X-gal and neutral red revealed patchy distribution of blue NSCs (Fig. 7A,B). No NSCs were detected by X-gal staining in liver (Fig 7C), kidney (Fig 7D), heart (Fig 7E) or lung (Fig 7F). No NSCs were detected in non-tumor bearing brains (data not shown). These results were confirmed on adjacent serial sections by beta-galactosidase immunohistochemistry. These results indicate that NSCs pass through the vasculature of most internal organs by day 4, with some trapping of cells in the spleen.

DISCUSSION

These results show that NSCs, and/or neuroprecursor cells, administered intravascularly in mice via a peripheral vein injection, demonstrate a remarkable capacity to rapidly localize to and infiltrate tumors within and outside the brain, with little infiltration of normal tissues. This phenomenon was observed for both neural (glioma and neuroblastoma) and non-neural (melanoma and prostate cancer) in both locations. NSCs were genetically modified to express the reporter gene lacZ, and in some cases the pro-drug activating enzyme, cytosine deaminase. These data support the potential of transgene-expressing NSCs to serve as effective delivery vehicles for gene products and/or viral vectors in gene-based therapies against brain tumors and a variety of systemic tumors. Engineered NSCs can be used in concert with other promising surgical, chemotherapeutic, radiotherapeutic, or genetic interventions.

NSCs can be used as delivery vehicles to target a variety of bioactive agents to tumors. In addition to pro-drug activating enzymes, such as cytosine deaminase (Huber et al., 1994; Aboody et al., 2000) and thymidine kinase (Chen et al., 1995) other therapeutic genes of potential utility include those encoding the following: molecules that promote differentiation of neoplastic cells (Kim et al., 1999); receptors for such trophins and/or their signal-transduction mediators; cell cycle modulators; apoptosis-promoting agents (Ehtesham et al., 2002); anti-angiogenesis factors (Joki et al., 2001); immune-enhancing agents; and oncolytic factors (Curiel *et al.*, 2000; Lam and Breakefield, 2001; Cusack and Tanabe, 2002). Furthermore, NSCs can serve to produce therapeutic vectors and through the dissemination of these cells achieve more efficacious delivery of viral vector-encoded therapeutic gene products throughout the tumor (Lynch *et al.*, 1999; Chiocca, 2002; Hampl, 2003). These same migratory NSCs have been demonstrated to package and release replication-conditional viral vectors (Herrlinger et al.,

2000) to tumors *in vivo* following transplantation, thereby potentially magnifying the efficacy of viral-mediated gene transfer by serving as an "extended" delivery system of lethal genes to invasive tumor cells.

Cell-mediated vector delivery has been explored previously using retrovirus packaging cells to distribute retroviral vectors on site within brain tumors (Short et al., 1990; Kramm et al., 1995). In most studies, however, these packaging cells are derived from fibroblasts that do not migrate within the brain and poor gene delivery to tumors was found in neuropathologic assessment of clinical trials (Harsh et al., 2000). Glioma cells and endothelial cells (Lal et al., 1994) (Brown AB, 2003) also have been used as vehicles for gene delivery as they also migrate throughout a tumor. A major disadvantage of using glioma cells as vehicles is that the carrier cells themselves are tumorigenic and hence could contribute to the tumor burden. Endothelial cells can localize to a glioma (Brown AB, 2003) and are non-tumorigenic, but have not been observed to move beyond the vasculature of the main tumor mass. NSCs not only localize to sites of tumor growth when administered into the vasculature, but also appear to migrate out of the tumor neovasculature into the tumor parenchyma. These cells have a high migratory potential both within the tumor and toward new tumor foci without being tumorigenic themselves, and have proven to be capable of stably delivering bioactive agents expressed by transgenes into the proximity of tumor cells. These capacities have also made them very useful in therapeutic paradigms demanding global gene and cell replacement in animal models of neurodegeneration (Yandava et al., 1999). We have previously shown that NSCs genetically engineered to express at least one enzyme (CD) capable of converting a non-toxic compound (5-FC) to a chemotherapeutic drug (5-FU) can exert a therapeutic effect: as demonstrated by the significantly reduced size of resultant experimental brain tumors *in vivo* (Aboody et al., 2000).

This prototypical enzyme-pro-drug gene therapy strategy via the bystander effect represents one example of the many potential approaches to treating tumors with genetically engineered NSCs. Targeted localized chemotherapy, such as that suggested by our previously reported CD-pro-drug NSC therapeutic paradigm, could result in significant reduction of side effects and associated morbidity. The potential to administer these cells via the peripheral intravenous route with homing to primary tumors and potentially to metastatic neural and non-neural foci, offers a minimally invasive means of extended therapeutic delivery.

These studies indicate that NSCs retain both their migratory capacity and tropism for tumor cells, when administered through the peripheral vasculature. Additionally, they can be genetically modified *ex vivo* to express one or more genes of interest - and continue to stably express the transgene *in vivo*. NSCs can be genetically engineered to express reporter and therapeutic genes, as well as to produce virus vectors. Although this study does not focus on therapeutic efficacy, it provides proof-of-concept for the novel use of intravascularly administered genetically modified NSCs as a delivery vehicle to effectively target various tumors. Further studies are warranted to explore the clinically relevant implications for targeting therapeutic transgenes to both primary and metastatic intracranial and systemic tumors of both neural and non-neural origin.

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FIGURE LEGENDS

FIG. 1. Neural stem cell migration in response to conditioned media from glioma- and non-neural tumor cell lines in a 96-well modified Boyden chamber assay. Conditioned media from all tumor cell lines significantly stimulated the directional migration of the NSCs compared to the control (* = $p \leq 0.05$, t-test). As expected glioma cell lines (human U251 and rat CNS-1.GFP) significantly stimulated NSC migration (up to 2-fold compared to the control) but interestingly, conditioned media from murine melanoma B16/F10 and human prostate PC3 induced the highest chemotactic response (up to 3-fold). Endothelial cell (PAE/KDR) conditioned medium did not change the basal migration rate. Values shown are means – SE of quadruplicate determination from two independent experiments and expressed in percentage of the control migration (=100%).

FIG. 2. Intravascularly delivered transgene- expressing NSCs display tropism for extracerebral tumors. Five-ten X 10^6 tumor cells were implanted into the subcutaneous flank of adult nude mice. Three weeks later, when $\geq$ 2 mm tumors were established, 2-3 X 10^6 NSCs (§gal-labeled, C17.2 cells) were injected into the tail vein: [**A-B**, scale bar = 100 m] rodent CNS-1 glioma; [**C**, scale bar = 50 m] human U-251 glioma; [**D-F**, scale bar = 100, 80, 25 m respectively] SH-SY5Y human neuroblastoma. On day 4 following NSC tail vein administration, the animals were sacrificed, and tumor and organs harvested, post-fixed in 4% PFA, cryosectioned and stained. **A**: CNS-1 tumor tissue sections stained with X-gal and neutral red to examine distribution of C17.2 NSCs within the experimental subcutaneous tumor. **B**: Sister section processed for immunofluoresence using a Texas red-conjugated anti-§gal antibody (donor NSCs). [**C**] U-251 tumor tissue sections stained with X-gal and neutral red to examine donor

C17.2 NSC distribution within the experimental tumor. **D, E:** Low and high power views of SH-SY5Y tumor tissue sections stained with X-gal and neutral red to examine donor C17.CD2 NSC distribution within the experimental subcutaneous tumor. **F:** Sister section processed for double immunofluoresence using a Texas red-conjugated anti-§-gal antibody (donor NSCs).

FIG. 3. Intravascularly delivered NSCs localize to tumor vasculature. NSCs localize to tumor vasculature as seen at day 4 post NSC tail vein injection in U-251 human glioma subcutaneous flank tumor model. Tumor section was processed for double immunofluoresence using a Texas red conjugated anti-CD31 antibody (blood vessels) and FITC-conjugated anti- ßgal antibody (donor NSCs). [one panel, scale bar = 75 μm].

FIG. 4. Intravasculary delivered CD-expressing NSCs display tropism for extracerebral tumors within 30 minutes. Intravascularly delivered NSCs display tropism for extracerebral experimental tumors within 30 minutes. Five X 10^6 SH-SY5Y human neuroblastoma cells were implanted subcutaneously into the left and right flank of adult nude mouse. Three weeks later, when the tumors were well established, the left tumor mass was surgically removed (prior to NSC injection), and 2-3 X 10^6 NSCs were injected into the tail vein. Thirty minutes later, the animals were sacrificed by anesthesia overdose and right subcutaneous tumor mass (post-NSC injection) and organs harvested. [**A, C**] low and higher power view of left flank tumor tissue sections stained with X-gal and neutral red. Note lack of any X-gal positive cells. [**B, D-G**] Low and higher power views of right flank tumor tissue sections stained with X-gal and neutral red. Note ring like pattern of X-gal staining donor cells all along outer tumor border (black arrowheads and arrows). Scale bars **A, B** = 250 μm; **C, D** = 100 μm; **E** = 80 μm; **F** = 50 μm; **G**

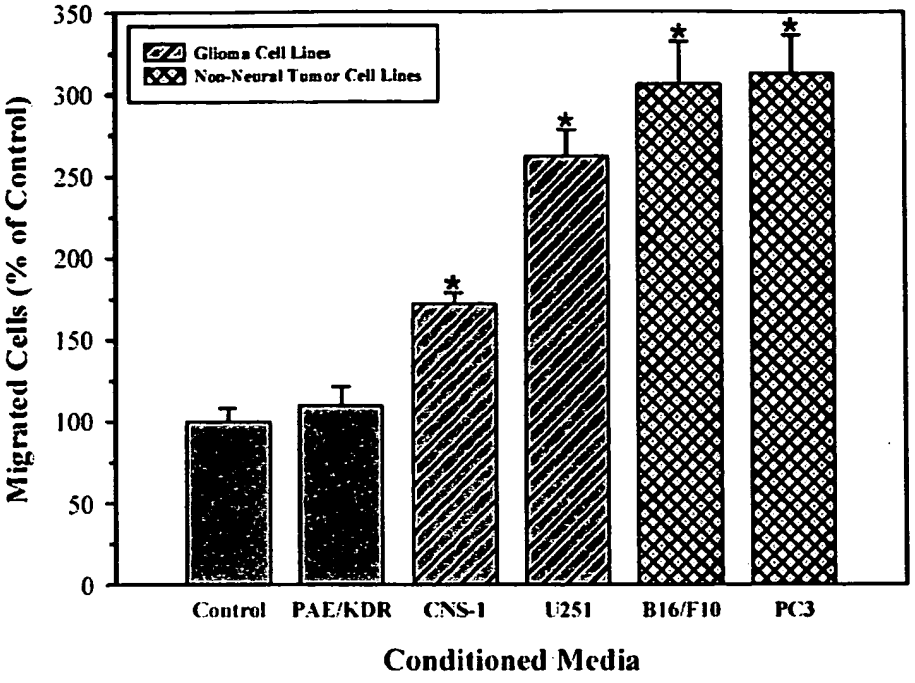
= 25 μm.

FIG. 5. Intravascularly delivered NSCs target both intracranial and subcutaneous human neural tumor within the same animal. Intravascularly delivered NSCs display tropism for extracerebral and intracerebral experimental neural tumors. 5-10 X 10^6 human U-251 glioma cells were implanted into the right subcutaneous flank and 8 X 10^4 human U-251 glioma cells were implanted into right frontal lobe of adult nude mouse. 2 weeks post frontal lobe implantation, 2-3 X 10^6 NSCs were injected into the tail vein. Four days following NSC tail vein administration, the animals were sacrificed by anesthesia overdose, and tumor and organs harvested, post-fixed in 4% PFA, cryosectioned, and stained. [**A, B**, scale bars = 50 μm, 25 μm respectively] low and high power views of intracranial tumor sections processed for immunofluorescence using Texas red-conjugated anti-ß-gal antibody (donor NSCs) and DAPI counterstain for all nuclei (white arrowheads delineate frontal lobe tumor, white arrows identify donor NSCs within tumor) [**C, D**, scale bars = 100 μm, 25 μm respectively] low and high power views of subcutaneous flank tumor sections processed for immunofluorescence using Texas red-conjugated anti-ß-gal antibody (donor NSCs) and DAPI counterstain (white arrowheads delineate tumor edge, white arrows identify donor NSCs within tumor).

FIG. 6. Intravascularly delivered CD-expressing NSCs target non-neural subcutaneous tumor. Five-ten X 10^6 human melanoma cells were implanted subcutaneously into the left flank of adult nude mice. Two-three weeks later, when the tumors were well established, 2-3 X 10^6 neural stem cells (C17.CD2s) were injected into the tail vein. Thirty minutes later, the animals were sacrificed and subcutaneous tumor and organs harvested. [**A, B**, scale bars = 100 μm, 25 μm

respectively]. Low and high power views of representative subcutaneous tumor section: DAPI filter to show tumor mass by high density of cell nuclei; Rhodamine filter to show Texas red conjugated anti- ßgal antibody (donor NSCs. Note distribution of NSCs throughout subcutaneous melanoma.

FIG. 7. Distribution NSCs through internal organs 4 days post NSC tail vein injection. Representative sections of internal organs from animals bearing a U-251 subcutaneous tumor model (see Fig. 2). [**A, B**, scale bars = 100 m, 50 m respectively]. Low and high powers views of representative section through spleen stained with X-gal (blue donor NSCs) and neutral red. Note patchy distribution of NSCs. [**C-F**, scale bars = 100 m]. Representative sections stained with X-gal and neutral red of [**C**] liver, [**D**] heart, [**E**] spleen and [**F**] lung. Note absence of blue donor NSCs.


Glioma Cell Lines
Non-Neural Tumor Cell Lines
Migrated Cells (% of Control)
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PC3
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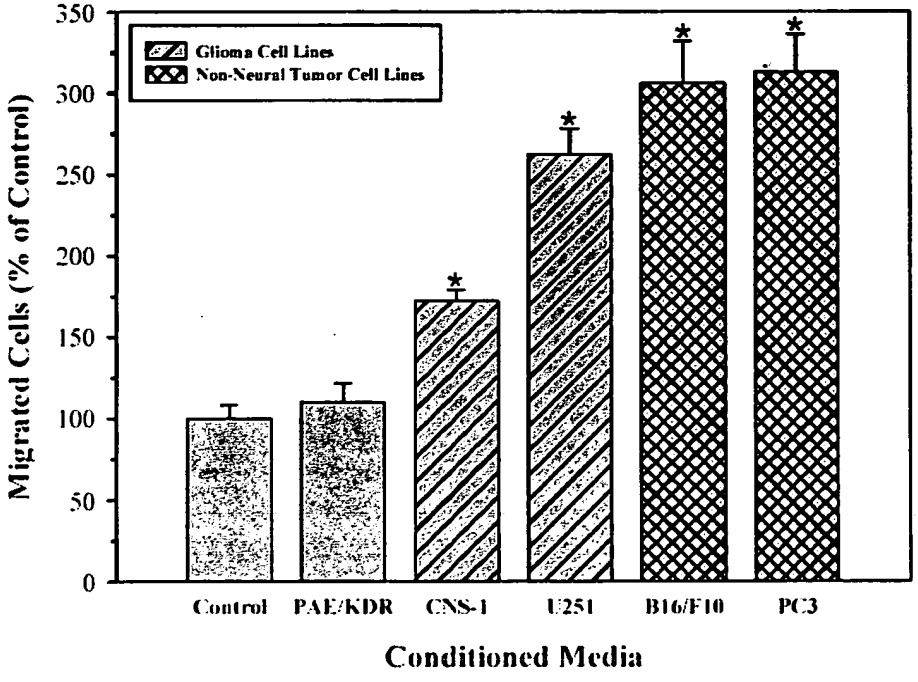
Glioma Cell Lines
Non-Neural Tumor Cell Lines
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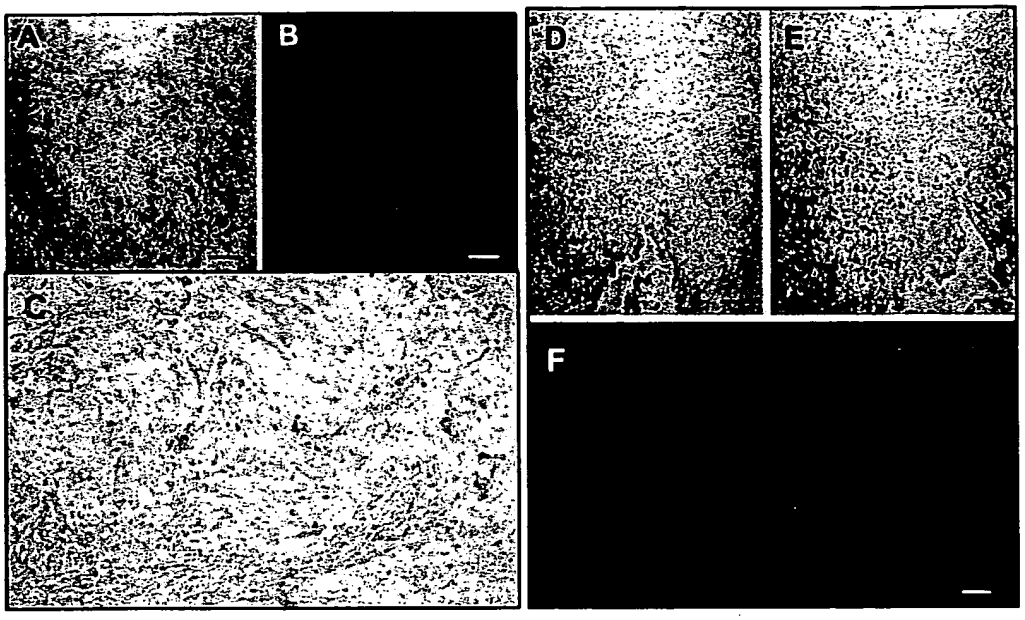
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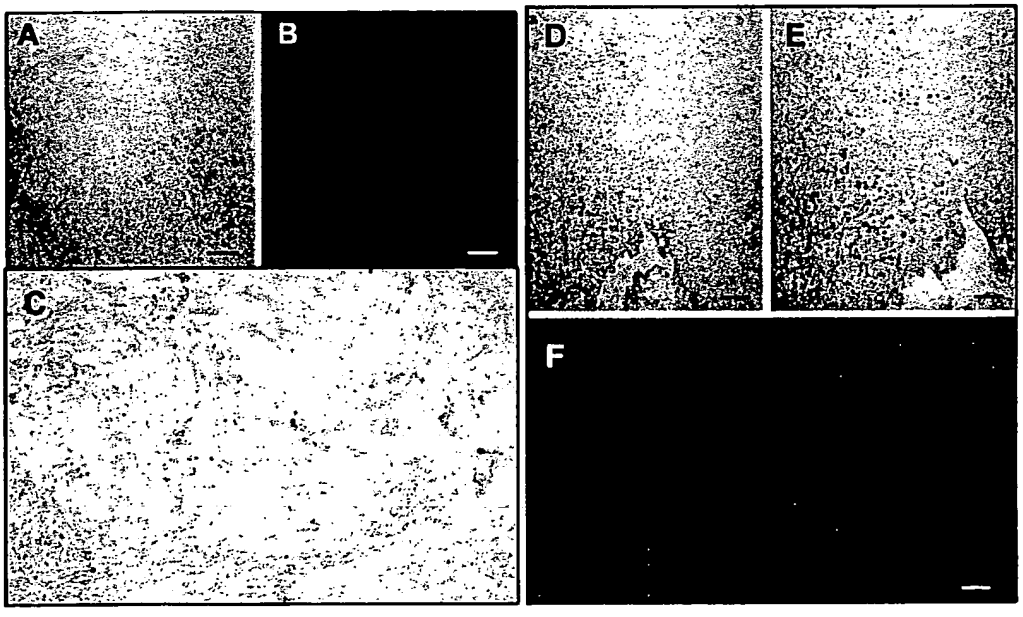
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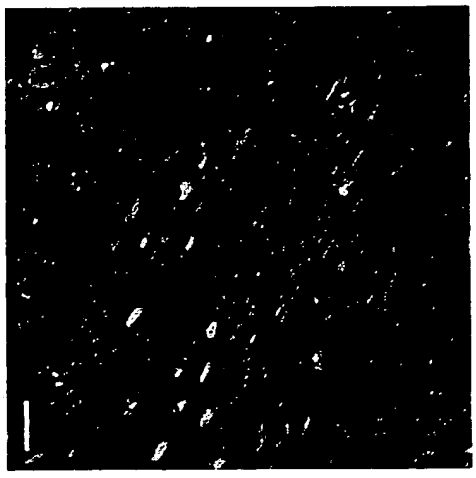

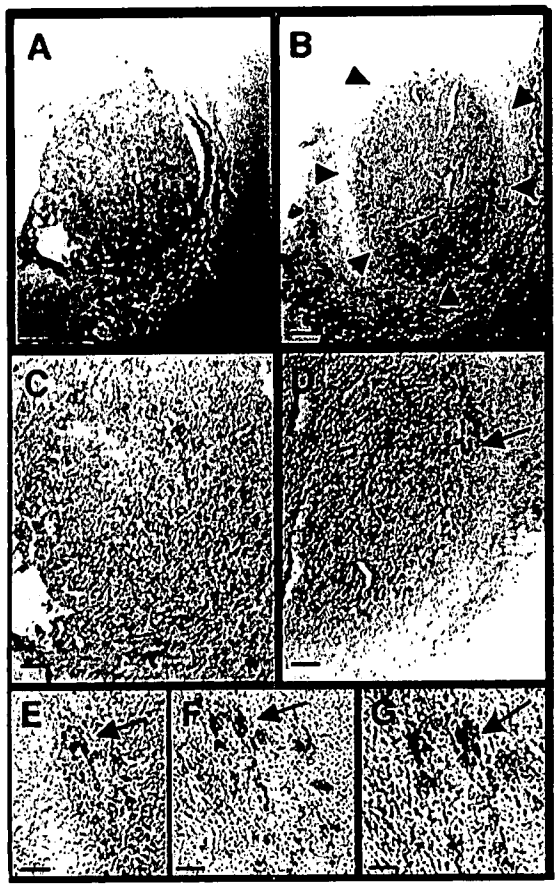
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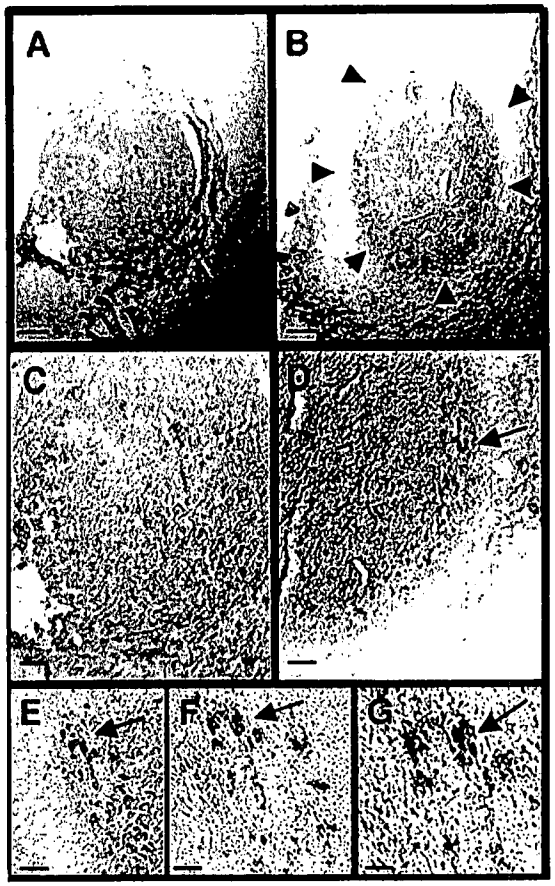
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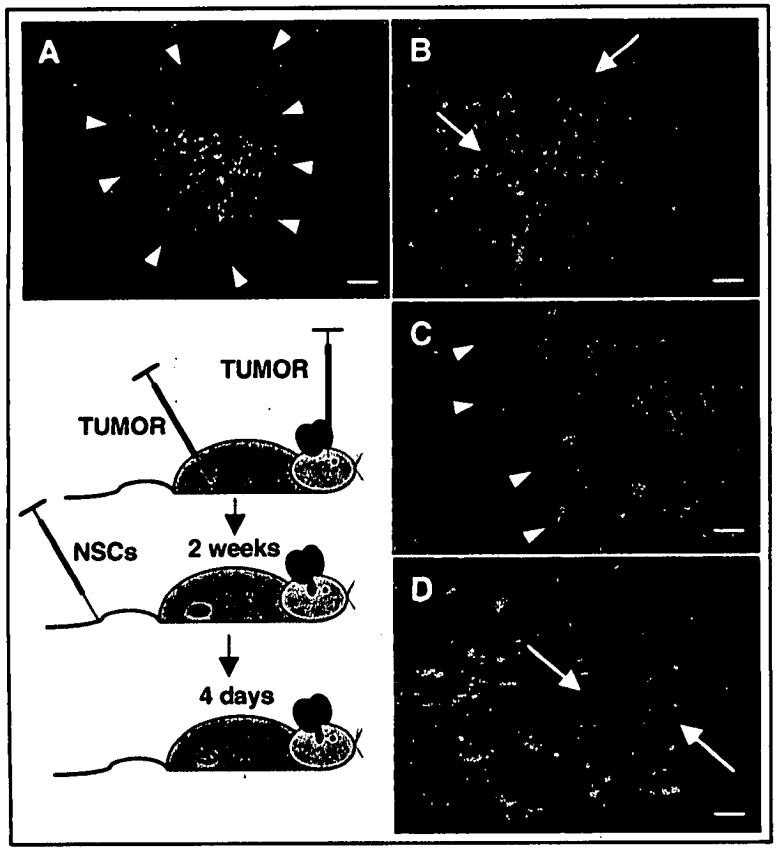
A
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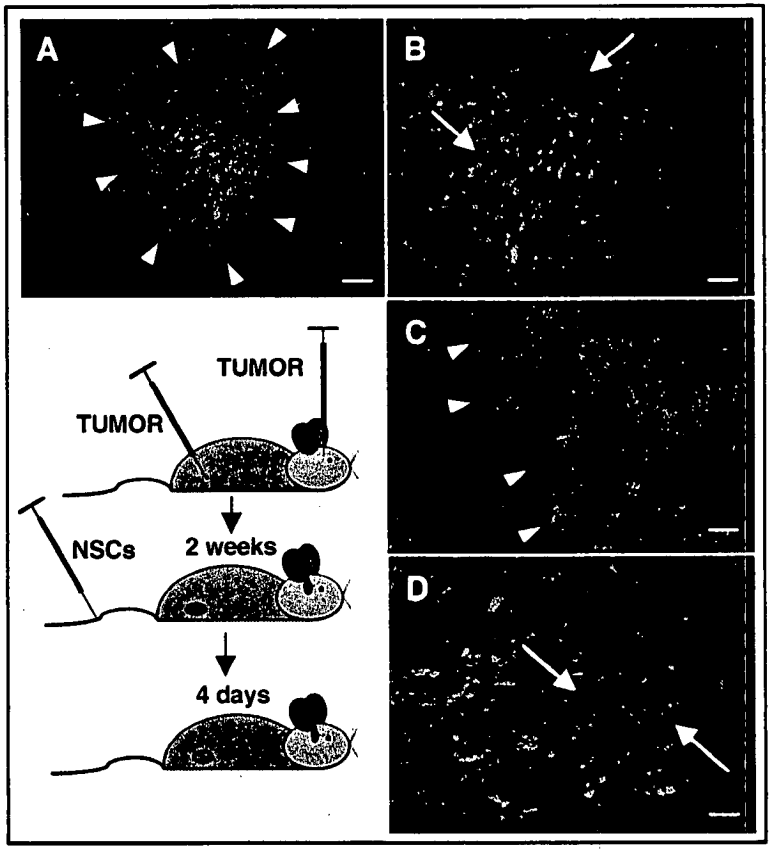
TUMOR
TUMOR
NSCs
2 weeks
4 days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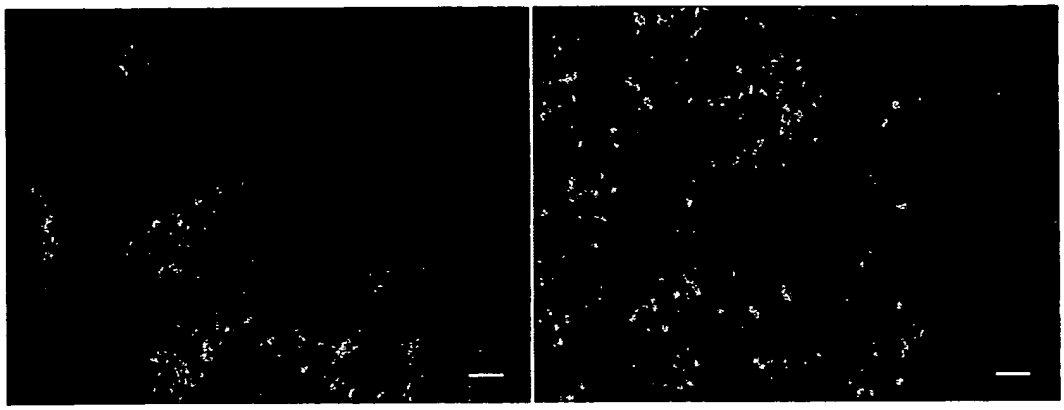

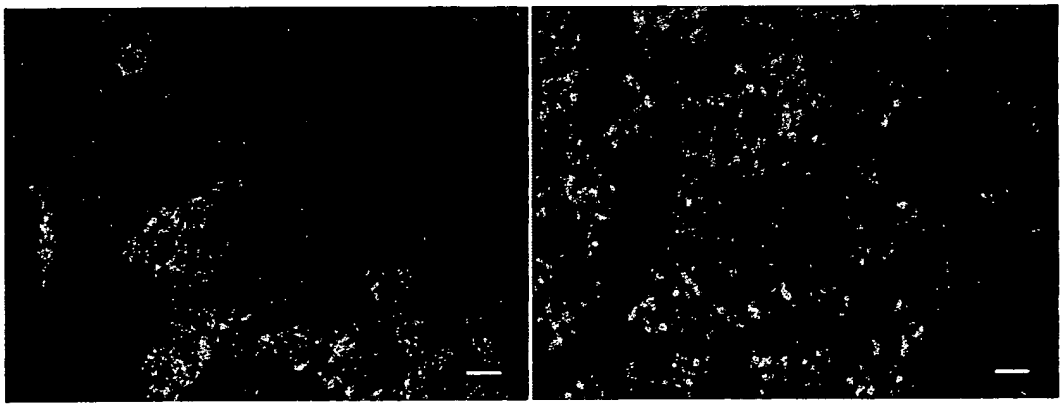

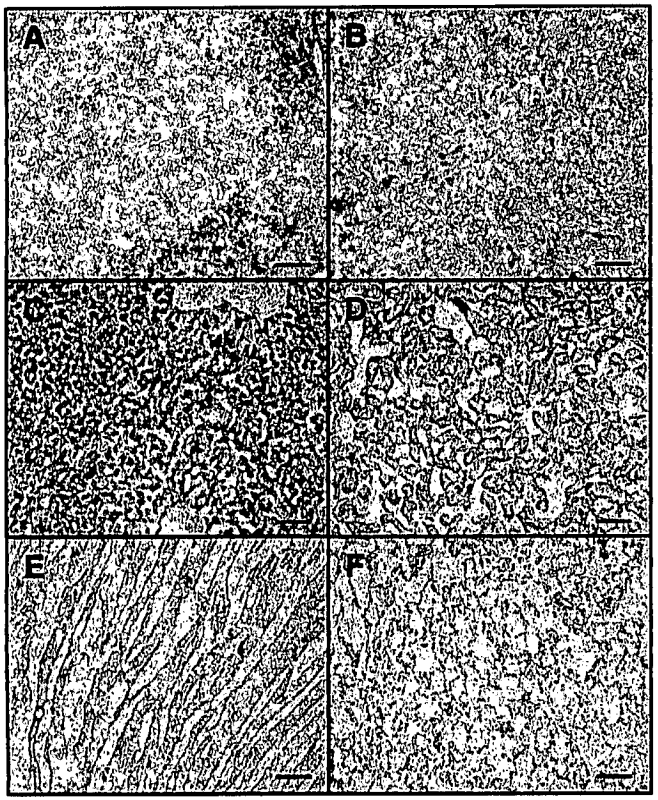
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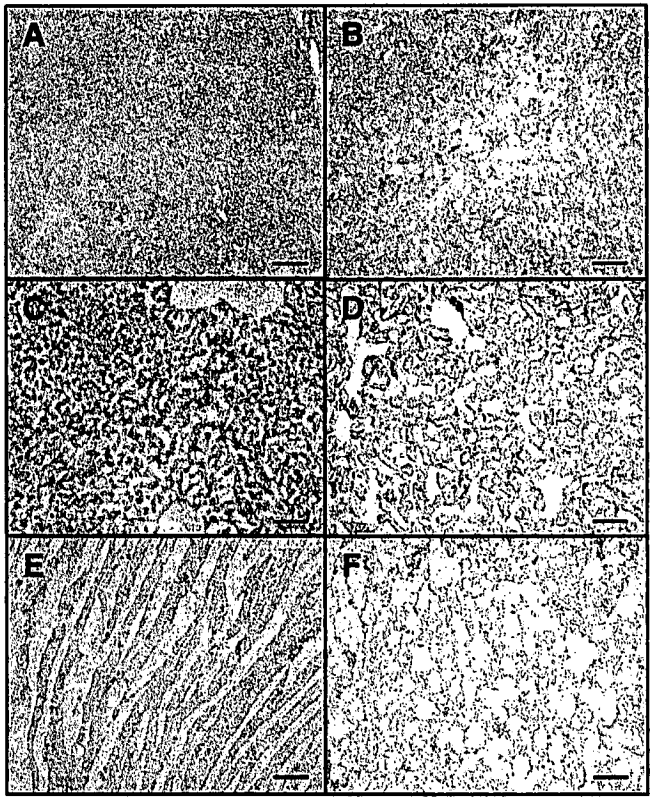
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